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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

MICHAEL LEBRUN ET AL

SERIAL NO. 08/945,144 **Art Unit: 1638**

FILED: OCTOBER 14, 1997 **Examiner: D. Kruse**

FOR: MUTATED 5-ENOL PYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE, GENE CODING FOR SAID PROTEIN AND TRANSFORMED PLANTS CONTAINING SAID GENE

Commissioner for Patents
Washington, D.C. 20231

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS BEING DEPOSITED WITH THE UNITED STATES POSTAL SERVICE AS FIRST-CLASS MAIL IN AN ENVELOPE ADDRESSED TO: COMMISSIONER FOR PATENTS, WASHINGTON D.C. 20231 ON THIS 28th DAY OF May 2002.

BY: Jean M. Marshall

DECLARATION OF RICHARD T. DEROSE, Ph.D.

I, RICHARD T. DEROSE, hereby declare:

1. I'm currently Director of the genomics platform in Evry, France, for Aventis CropScience S.A. and Director of the genomics platform for RhoBio, a joint venture between Aventis CropScience S.A. and Biogemma. Aventis CropScience S.A. is the assignee of the present patent application. I am familiar with the above patent application, and, in fact, helped draft some of the examples for the application, because I was involved in the experimental work.

2. I have a B.S. in microbiology with a molecular biology option from Clemson University, South-Carolina, and a Ph.D. in microbiology and immunology from Wake Forest Bowman Gray School of Medicine, North-Carolina. After my doctorate at Wake Forest, I worked at Texas A&M University working with Drs. Timothy Hall and Terry Thomas in the field of plant molecular biology for five years. In 1991, I joined then Rhône-Poulenc Agrochimie (RPA) in Lyon, France, where I started to work as "bench scientist" in a herbicide tolerance program, and moved to the position of scientific coordinator in 1994, before joining my current position in 1998, for RPA, which became Aventis CropScience S.A. through a merger with

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AgrEvo in 2000. I worked directly with genes for expressing glyphosate tolerant EPSPS enzymes and the general area of glyphosate tolerance during the period of 1991 to 1994, and, thereafter, from about 1997 to 1998.

3. 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is a key enzyme in the aromatic amino acid biosynthetic pathway in microorganisms and plants. It is the sixth enzyme on the shikimate pathway, which is essential for the synthesis of aromatic amino acids and of almost all other aromatic compounds in algae, higher plants, bacteria, and fungi. EPSPS catalyzes a reversible transfer of the enolpyruvyl group from phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P) forming the products 5-enolpyruvylshikimate-3-phosphate (EPSP) and inorganic phosphate (Pi).

4. Attached as Exhibits 1 and 2 are graphical depictions of a three-dimensional structure of the EPSPS enzyme from Escherichia coli that had been determined by crystallographic techniques and was published in 1991 by Stallings W.C. *et al.* in *Proc. Natl. Acad. Sci. USA*: Vol. 88, No. 11, 5046 - 5050, June 1, 1991, "Structure and topological symmetry of the glyphosate target 5-enolpyruvylshikimate-3-phosphate synthase: A distinctive protein fold." The above structure has been confirmed more recently. See Schönbrunn, E. *et al.*, *Proc. Natl. Acad. Sci. USA*: Vol. 98, No. 4, 1376 - 1380, February 13, 2001, "Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail." (Exhibit 3)

5. Glyphosate is the most extensively used herbicide for broad-spectrum control of weeds. Glyphosate acts by inhibiting EPSPS. Glyphosate's inhibition of EPSPS is a slowly reversible reaction, which is competitive versus PEP and uncompetitive versus S3P.

6. All known plant EPSPS enzymes contain a highly conserved region in the area of residue number of about positions 90 - 110. That conserved region was well defined and accepted in the art by June 1995. Thus, in United States Patent 4,971,908 to Kishore *et al.* titled "Glyphosate-tolerant 5-enolpyruvyl-3-phosphoshikimate synthase" and filed April 22, 1988, it was stated:

> FIG. 2 shows the amino acid sequence for EPSP synthase from various plant, bacteria and fungal species. Inspection of the sequences and alignment to maximize the similarity of sequence reveals a region of highly conserved amino

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acid residues (indicated by the box) in the region of the *E. coli* EPSP synthase mutant where the alanine for glycine substitution occurred. Indeed, all EPSP synthase enzymes reported in the literature and in the present specification reveal a glycine at this position in this highly conserved region.

See also, for example, the following U.S. Patents which refer to this domain as being highly conserved: United States Patent 5,859,347 (" in the highly conserved region having the sequence: -L-G-N-A-G-T-A- located between positions 80 and 120 in the mature wild-type EPSP synthase amino acid sequence"); United States Patent 5,145,783 (See Abstract: "The glyphosate-tolerant EPSP synthases are prepared by substituting an alanine residue for a glycine residue in a conserved sequence found between positions 80 and 120 in the mature wild-type EPSP synthase."); United States Patent 5,310,667 (See Abstract: "The glyphosate-tolerant EPSP synthases are prepared by substituting an alanine residue for a glycine residue in a first conserved sequence found between positions 80 and 120, and either an aspartic acid residue or asparagine residue for a glycine residue in a second conserved sequence found between positions 120 and 160 in the mature wild type EPSP synthase."). Although the above are patents, and not peer-reviewed literature, they do, in this case, represent the common understanding of the art.

7. Figure 2 of United States Patent 4,971,908 shows that Petunia, Tomato, Arabidopsis, Soybean and Maize have a common conserved region comprising:

(Q)-L-(F)-L-G-N-A-G-T-A-M-R-P-L-T-A-A-V-.

By 1995, the following sequence was also shown:

Brassica napus: E-L-Y-L-G-N-A-G-T-A-M-R-P-L-T-A-A-V- (ACCESSION: CAA35839)

Since 1995, the following species have been shown to have the following sequences:

Oryza sativa: Q-L-F-L-G-N-A-G-T-A-M-R-P-L-T-A-A-V- (ACCESSION: BAB61062; BAA32276)

Eleusine indica: Q-L-F-L-G-N-A-G-T-A-M-R-P-L-T-A-A-V- (ACCESSION: CAD01096)

Dicliptera chinensis: Q-L-F-L-G-N-A-G-T-A-M-R-P-L-T-A-A-V- (ACCESSION: AAL27697)

Lolium rigidum: K-L-F-L-G-N-A-G-T-A-M-R-P-L-T-A-A-V- (ACCESSION: AAK20397)

It is thus apparent that at least essentially all EPSPS enzymes in plants have a common conserved region, and this was recognized by the art by 1995.

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8. It was also know from points set forth in paragraphs 1-7, above, that the conserved region was in a position previously known to be important for catalysis or substrate binding, and the area where substitutions of wild-type amino acid residues for other amino acid residues can provide the proper combination of resistance to glyphosate while permitting the normal necessary catalytic functions to proceed.. See, for example, Padgette S.R., *J Biol Chem* 1991 Nov 25; 266(33):22364-9 "Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site," where it is noted that "This highly conserved region is critical for the interaction of the phosphate moiety of phosphoenolpyruvate with EPSPS." In United States Patent 4,971,908 the patentees continued that "It has been found that the alanine for glycine substitution can be introduced into this highly conserved region of other wild-type EPSP synthase enzymes to yield glyphosate-tolerant EPSP synthase enzymes." And, then pointed out:

> Hence, in one aspect the present invention provides glyphosate-tolerant EPSP synthase enzymes and a method for producing such enzymes which comprises substituting an alanine residue for the second glycine residue in the highly conserved region having the sequence:
>
> -L-G-N-A-G-T-A-
>
> located between positions 80 and 120 in the mature wild-type EPSP synthase amino acid sequence. In most cases the above sequence will be located between positions 90 and 110 in the mature EPSP synthase.

9. See also Stallings W.C. *et al.*, *supra*. The authors there noted in the Abstract that: "The topological threefold symmetry and orientation of each of the two observed globular domains may direct the binding of substrates and inhibitors by a helix macrodipole effect and implies that the active site is located near the interdomain crossover segments. The structure also suggests a rationale for the glyphosate tolerance conferred by sequence alterations."

10. In fact, it was recognized in 1995, as it is today, that plant EPSPS enzymes are effectively interchangeable. Thus, one can eliminate or disable the EPSPS enzyme in a plant species, and substitute an EPSPS enzyme from another plant species, and the plant will function. This is demonstrated by the fact that, as shown in this application, as well as in many prior publications, an EPSPS enzyme from a first plant species mutated to be glyphosate tolerant can

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be introduced into a plant of a second different species than the first species, and yet the glyphosate will disable the wild-type or natural enzyme in the second species, but the plant will continue to function, because the mutated glyphosate-tolerant EPSPS enzyme of the first plant species continues to perform the normal EPSPS catalytic function in that plant. Even as of today, I am not aware of any report of a heterologous plant EPSPS enzyme that did not function in a plant of a different species in the same manner as the wild-type or natural EPSPS enzyme.

11. The plant EPSPS enzymes were viewed to be essentially interchangeable because all have the very highly conserved active site in the area of residue numbers of about 90 - 110 described above.

12. In order to refer to an amino acid residue in the EPSPS enzyme, it was, and remains, conventional in the art to refer to the residue followed by a number, which may or may not reflect the exact position of the residue in the enzyme, but differentiates the residue from other residues in the enzyme. In other words, the number associated with a residue does not identify a position, which is utterly irrelevant, but a specific residue in the EPSPS enzyme, which is critical. For example, as of 1995, the best know and most recognized EPSPS enzyme mutation was identified interchangeably as "Gly96Ala," or "Gly97Ala" or "Gly101Ala," and it was generally understood and recognized that both these designations referred to a particular Glycine residue in the conserved region without regard to what might be the exact specific location. This was true, even though the actual Glycine residue may be the 96th , 97th, 101st or, in the case of soybean EPSPS, the 104st residue in a particular EPSPS enzyme.

13. The key problem was and remains that introducing any EPSPS enzyme into a plant which has had its amino acid sequence mutated or changed usually produces either -- (a) no glyphosate tolerance because the changed residue does not resist glyphosate inhibition, or (b) the enzyme has been so changed that the EPSPS enzyme no longer can continue its normal catalytic activity, thus resulting in a damaged plant. Modification of the EPSPS enzyme was and is easy, and there were multiple techniques available to make mutated EPSPS enzymes. The problem in the art was to make the special combination of (a) choosing a particular residue to be modified; (b) choosing the substituted residue from the other available nineteen amino acids; and, potentially, (c) combine this substitution with other substitutions following steps (a) and (b),

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that would produce a modified EPSPS enzyme that would resist glyphosate inhibition, but continue to perform the normal catalytic function required to sustain the life of a plant.

14. The Examiner stated:

> 14. Claim 42 is rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Specifically, Applicant has only described a modified maize EPSPS encoding polynucleotide having the claimed modification. Applicant does not describe any other plant EPSPS encoding polynucleotides having the claimed modifications. Hence, it is unclear from the instant specification that Applicant was in possession of the invention as broadly claimed.

15. In fact, given that, at the time the application was filed, all known plant EPSPS enzymes were known to have the identical conserved region and that it was apparent that any plant EPSPS enzyme was essentially interchangeable with any other EPSP enzyme, the only possible conclusion that any person of ordinary skill in the art could reach is that the disclosed EPSPS mutations would be applicable to every plant EPSPS enzyme. The application itself states that "The present invention relates to a new [EPSPS] which displays increased tolerance with respect to herbicides which are competitive inhibitors with respect to phosphoenolpyruvate (PEP) of EPSPS activity. This more tolerant EPSP synthase possesses at least one 'threonine by isoleucine' substitution." Thereafter, the application speaks generally to the EPSPS enzyme and plants, without any limitation to the particular plant source for the plant EPSPS.

16. Further, the Examiner states that "Applicant does not describe any other plant EPSPS encoding polynucleotides having the claimed modifications." That is not correct, because, as noted above, all known plant EPSPS enzymes have the same residues in the conserved region, and the EPSPS enzymes are essentially interchangeable. Therefore, the polynucleotides necessary to mutate the DNA can be the same as described in the application. Thus, the application does disclose "plant EPSPS encoding polynucleotides having the claimed modifications" which will be effective with any plant EPSPS.

17. I note also the Examiner's reference that "See *University of California V. Eli Lilly and Co.*, 43 USPQ2d 1398 (Fed. Cir. 1997), which teaches that the disclosure of a process for obtaining cDNA from a particular organism and the description of the encoded protein fail to

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provide an adequate written description of the actual cDNA from that organism which would encode the protein from that organism, despite the disclosure of a cDNA encoding that protein from another organism." I am not qualified to discuss patent law, but, will note that the above description of the case has no application here. The application here discloses the cDNA of the maize EPSPS, but that is not the point of the application. The application clearly describes the mutation of the EPSPS - encoding gene to produce a glyphosate tolerant mutated EPSPS enzyme. The same mutations can be made in any plant EPSPS gene, because, as noted above, they all produce basically the same EPSPS enzyme structure and function, and by 1995, there were many techniques known to the art to accomplish the mutations.

18. The Examiner further states that:

> Claims 42, 45, 46 and 50-53 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabling for an isolated polynucleotide of maize origin encoding a modified 5-enolpyruvylshikimate-3-phosphate synthase having the claimed modifications, vectors, transgenic plants and plant cells comprising said DNA sequence, and a method of protecting plants comprising said transgenic plants, does not reasonably provide enablement for all isolated polynucleotides of plant origin encoding a modified 5-enolpyruvylshikimate-3-phosphate synthase having the claimed modifications, vectors, transgenic plants and plant cells comprising said DNA sequence. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

I disagree, because that assumes the absence of any skill in the art, which, if fact, was as sophisticated and fast-moving as any art. Again, given the knowledge to one skilled in the art of the conserved region and the essential interchangeability of the plant EPSPS enzymes within plant species, and the description in the application of the one actual process that had been used to mutate the EPSPS, any person of ordinary skill in the art would have been able to use the same identical oligos and processes to mutate virtually any plant-derived EPSPS gene to make the claimed EPSPS enzymes. I note that this would hold true even if the position of the Thr and Pro residues was not exactly at the 102st and 106st residue locations of the mature enzyme.

19. Thus, the oligo -

5'- GGGGAATGCTGGAATCGCAATGCGGTCCTTGACAGC - 3'

described at page 20 of the application will make the Thr102Ile/Pro106Ser mutations in any

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publically disclosed monocot EPSPS gene, and under appropriate conditions most likely this oligo would function to create these mutations any publically disclosed dicot EPSPS gene, without regard to whether the Thr and Pro actually occupy positions 102 and 106 or some other position.

20. This is demonstrated by the following chart:

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      Oligo ~~~~~~~~GG GGAATGCTGG AATCGCAATG CGGTCCTTGA CAGC~~~~~~
      Maize CTCTTCTTGG GGAATGCTGG AACTGCAATG CGGCCATTGA CAGCAGCTGT
Goosegrass1 CTCTTCTTGG GGAATGCTGG AACTGCAATG CGATCATTGA CAGCAGCCGT
Goosegrass2 CTCTTCTTGG GGAATGCTGG AACTGCAATG CGACCATTGA CAGCAGCCGT
  rye grass CTCTTCTTGG GGAACGCTGG AACTGCGATG CGGCCATTGA CGGCTGCTGT
       Rice CTCTTCTTGG GGAACGCTGG AACTGCAATG CGACCATTGA CAGCAGCCGT
D chinensis CTTTTCTTGG GGAATGCAGG GACTGCTATG CGACCATTGA CGGCTGCTGT
     Petunia CTGTTCCTTG GAAATGCAGG AACAGCAATG CGGCCACTAA CAGCAGCAGT
     B napus TTGTACCTTG GGAATGCAGG AACAGCCATG CGTCCACTCA CCGCTGCAGT
     tobacco CTATTCCTTG GAAATGCAGG AACAGCAATG CGGCCATTGA CGGCAGCAGT
      tomato CTATTCCTTG GAAATGCAGG AACAGCAATG CGTCCGTTGA CAGCAGCAGT
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21. In the above chart, the nucleic acids underlined in the "Oligo" line are the mutations that cause the Thr102Ile/Pro106Ser modification in the EPSPS enzyme. The nucleic acids for the different plant species are as reported in the literature for the relevant portion of the plant's EPSPS coding region. I note that the above constitute all the EPSPS coding regions that I have been able to identify in the literature. The "GG" and the "GC" at the respective ends of the oligo are recognized clamps for introducing the mutated oligos into genomic EPSPS coding region by way of standard PCR techniques.

22 As is immediately apparent, the referenced oligo will as clearly mutate the other known monocot EPSPS coding sequences as the maize EPSPS coding region, because the functional coding sequence is identical.

23. Further, there is only one minor difference shown in the relevant coding regions of the dicot plants, and that is that the dicot plants have a "TG" rather than a "GG" clamp on one end. The other end shares the "GC" clamp of the oligo. As would be recognized by an ordinarily

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skilled worker in the art, the oligo should work in a standard PCR reaction to mutate that coding region by testing a range of temperatures and magnesium concentrations of the type typically encountered in such processes. A person of ordinary skill in the art in 1995 also knew how to modify known oligos to make them useful in mutating specific DNA sequences. The skilled person would, therefore, modify the specific oligo in the application in one of several ways to avoid changing the PCR conditions. For example, the person would perfectly align the 5' and 3' ends of the oligo to the sequence of the EPSPS in issue. Or as another equally acceptable known alternative, the person skilled in the art would conform the oligo to the EPSPS in question. Thus, to mutate the tomato EPSPS disclosed in the public domain (paragraph 20 above), the skilled person would either change the first G of the oligo to a T, or just remove the first G of the oligo. Any of these minor changes would allow the oligo of the application to mutate the EPSPS, and all these techniques were well-developed by 1995.

24. I also refer to another reference, International Patent Application WO 00/66746, published November 9, 2000. That reference discloses the mutation of a rice EPSPS coding region to provide the Thr102Ile/Pro106Ser modification in the rice genomic DNA. As shown at page 13 of the reference, the workers used Sequence ID No 7 to provide the Thr102Ile/Pro106Ser modification. The oligo, Sequence ID No. 7 is functionally identical to the oligo of the present application and provides the same mutation of the EPSPS coding region in the rice genomic DNA. This is illustrated by the chart below:

APPLICATION OLIGO:

G GGG AAT GCT GGA ATC GCA ATG CGG TCC TTG ACA GC

G N A G I A M R S L T

WO 00/66746 SEQ. ID NO. 7:

GG AAC GCT GGA ATT GCA ATG CGA TCA TTG ACA GCA GCC GTG ACT GC

N A G I A M R S L T A A V T

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I note that because of codon degeneracy, there are multiple codons that can be used to encode a given residue, and, given the presence of multiple clamping sites, that a worker has a choice of the length of the transforming oligos.

Therefore, this reference effectively confirms that the present oligo of the application will mutate the rice genomic DNA to provide the Thr102Ile/Pro106Ser modification, whether or not the mutated positions are at positions 102 and 106 of the rice genomic DNA.

25. The Examiner also states that:

> Applicant argues that the specification states expressly that the modified DNA molecule may be of plant origin (page 4, lines 13-14 of the Remarks). Applicant argues that the domain of the EPSPS enzyme overlapping relative positions 102 to 106 (of the maize sequence) has been recognized in the prior art as being highly conserved in plants (page 4, lines 24-25 of the Remarks). In addition, Applicant argues that the prior art teaches that EPSPS enzymes in plant species have significant similarity, citing USP 5,310,667, figure la-b, and that it would not have required undue trial and error experimentation for one of ordinary skill in the art to isolate all DNA sequences encoding an EPSPS gene and modify them as claimed (pages 5-6 of the Remarks). The Examiner responds, that the statement of intent to modify other plant DNA molecules does not necessarily enable the specification within the scope of the instant claimed invention. It is noted that Applicant claims modification of an EPSPS enzyme at positions 102 and 106, not relative positions. In addition, Applicant's reliance upon the teachings of others directed to different mutations of the EPSPS enzyme is irrelevant. The '667 patent teaches modification of the petunia, tomato, *Brassica* and maize genes encoding the same modification and producing the same glyphosate tolerance. Applicant has only taught that modification at positions 102 and 106 of the maize EPSPS enzyme produces a glyphosate tolerant enzyme.

26. I disagree with the statement that "Applicant claims modification of an EPSPS enzyme at positions 102 and 106." The references to 102 and 106 in the application clearly correspond to the numbering sequence of Sequence ID No. 3 in the application. The application at page 3 clearly states that the numerical numbering sequence is "relative to the gene from which it is derived." Further, any person skilled in the art reading the application would in 1995 have understood, and would understand today, that it is a particular residue that is being modified, not a "position." In all the years of working in this area, I have never heard of anyone ever attempting to modify a "position" rather than a residue. As I noted above, the "102" and

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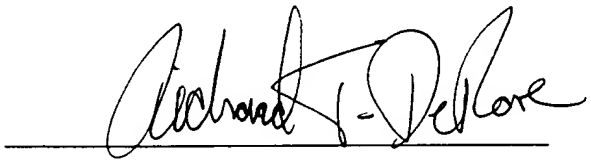
"106" designations merely specifically identify the particular Thr and Pro that is modified, not a position to be mutated. The latter makes no technical sense, and, therefore, could never be viewed as the understanding of one skilled in the art. Because the Thr and Pro residues may, for a variety of reasons, not be the 102nd and 106th amino acid residues of the particular enzyme, but would clearly be identifiable by any person of ordinary skill in the art, because the conserved region of plant EPSPS enzymes mandates that the Thr102Ile/Pro106Ser modifications can only refer to one and only one Thr, and one and only one Pro.

27. I note that in the description and the proposed claims of WO 00/66746, the workers simply refer to the above mutations as being in the "conserved region" rather than identifying them by number (see, for example, proposed claim 5 at page 58), which is consistent with my opinion that a person of ordinary skill in the art has and continues to recognize the conserved region of EPSPS coding sequences and that the position numbering merely identifies the particular residue rather than the position.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: April 4, 2002



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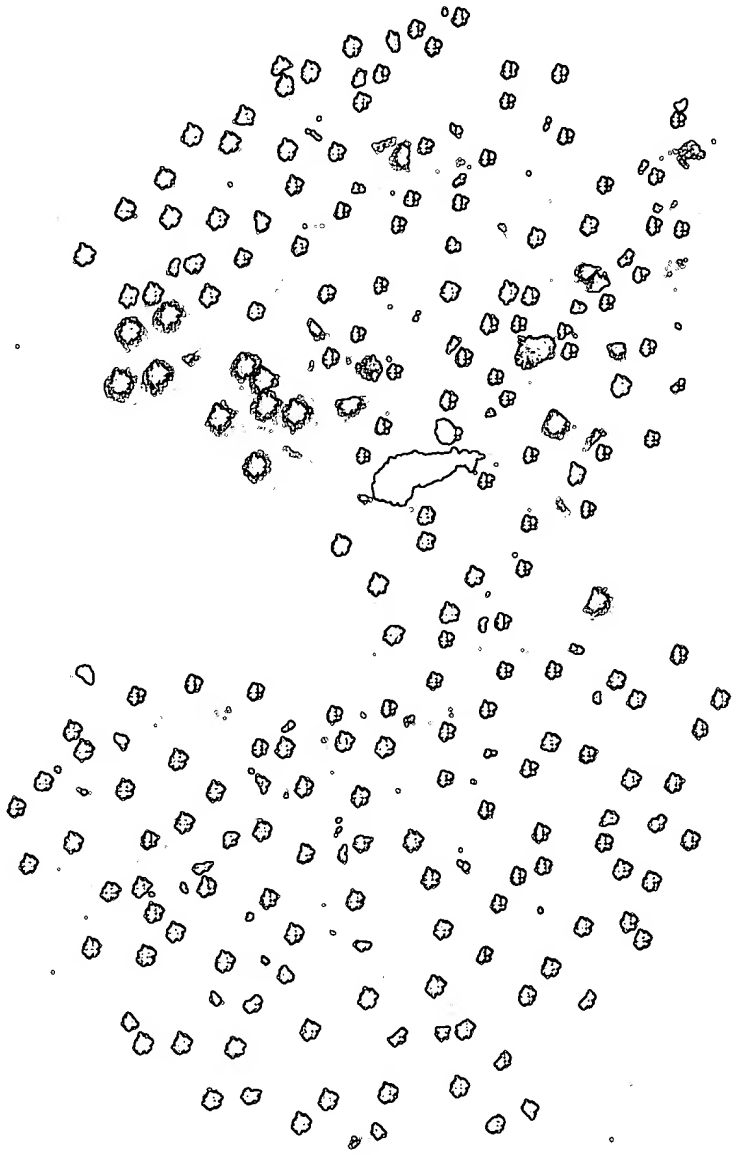

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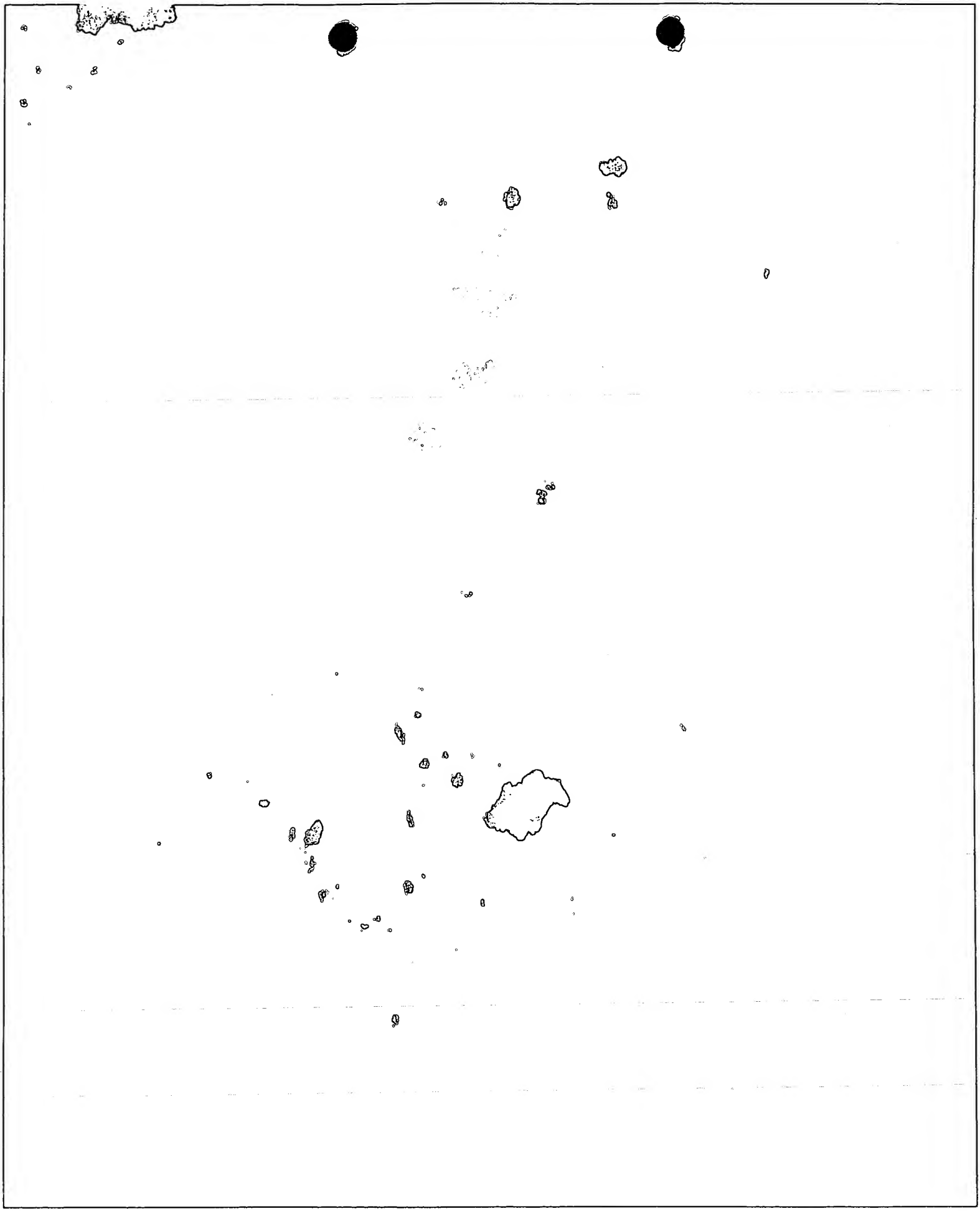


Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail

Ernst Schönbrunn*†‡, Susanne Eschenburg†§, Wendy A. Shuttleworth¶, John V. Schloss*, Nikolaus Amrhein‖, Jeremy N. S. Evans¶, and Wolfgang Kabsch§

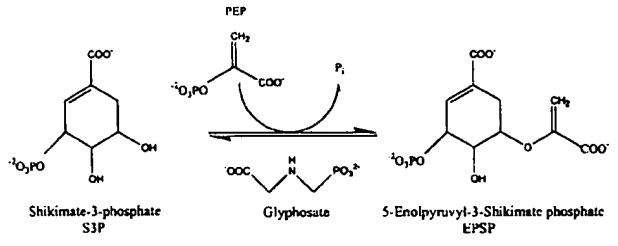
*Department of Medicinal Chemistry, University of Kansas, Lawrence, KS 66045; §Max Planck Institute for Medical Research, Department of Biophysics, D-69120 Heidelberg, Germany; ¶School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4660; and ‖Institute of Plant Sciences, Swiss Federal Institute of Technology, CH-8092 Zurich, Switzerland

Edited by Gregory A. Petsko, Brandeis University, Waltham, MA, and approved December 13, 2000 (received for review August 25, 2000)

Biosynthesis of aromatic amino acids in plants, many bacteria, and microbes relies on the enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase, a prime target for drugs and herbicides. We have identified the interaction of EPSP synthase with one of its two substrates (shikimate 3-phosphate) and with the widely used herbicide glyphosate by x-ray crystallography. The two-domain enzyme closes on ligand binding, thereby forming the active site in the interdomain cleft. Glyphosate appears to occupy the binding site of the second substrate of EPSP synthase (phosphoenol pyruvate), mimicking an intermediate state of the ternary enzyme-substrates complex. The elucidation of the active site of EPSP synthase and especially of the binding pattern of glyphosate provides a valuable roadmap for engineering new herbicides and herbicide-resistant crops, as well as new antibiotic and antiparasitic drugs.

The enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19) is the sixth enzyme on the shikimate pathway, which is essential for the synthesis of aromatic amino acids and of almost all other aromatic compounds in algae, higher plants, bacteria, and fungi (1–3), as well as in apicomplexan parasites (4). Because the shikimate pathway is absent from mammals (2, 3), EPSP synthase is an attractive target for the development of new antimicrobial agents effective against bacterial, parasitical, and fungal pathogens. A valuable lead compound in the search for new drugs and herbicides is glyphosate, which has proven as potent and specific inhibitor of EPSP synthase (5). Glyphosate is successfully used as a herbicide, being the active ingredient of the widely used weed control agent Roundup, and was recently shown to inhibit the growth of the pathogenic parasites *Plasmodium falciparum* (malaria), *Toxoplasma gondii*, and *Cryptosporidium parvum* (4).

EPSP synthase catalyzes the transfer of the enolpyruvyl moiety from phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P) forming the products EPSP and inorganic phosphate (Scheme 1) (1, 6). The reaction is chemically unusual because it proceeds via C–O bond cleavage of PEP rather than via P–O bond cleavage (7) as in most PEP-utilizing enzymes. Glyphosate inhibits EPSP synthase in a slowly reversible reaction, which is competitive versus PEP and uncompetitive versus S3P (5, 8, 9).



EPSP synthase (M_r 46,000) folds into two similar domains (10) (Fig. 1), each comprising three copies of a $\beta\alpha\beta\alpha\beta\beta$-folding unit. The only other enzyme known to exhibit this architecture is the mechanistic homologue UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA, EC 2.5.1.7) (11), which catalyzes the transfer of the intact enolpyruvyl moiety of PEP to a sugar nucleotide. MurA is essential for the synthesis of the bacterial cell wall and is the target of the broad spectrum antibiotic fosfomycin (12). On sugar nucleotide binding, MurA undergoes large conformational changes leading to the formation of the active site (13–16).

Although EPSP synthase has been extensively studied over more than three decades (17), conclusions on the enzyme mechanism (8, 9, 17, 18) and especially on the mode of action of the herbicide glyphosate (9, 17, 18) remained controversial. Up to now, the three-dimensional structure of EPSP synthase was only known in its unliganded form (10, 17), which does not reveal the active site of the enzyme. We have co-crystallized EPSP synthase with S3P and glyphosate as well as with S3P alone and determined the structures of these complexes at 1.5 and 1.6 Å resolution, respectively.

Methods

Catalytically competent EPSP synthase from *Escherichia coli* was purified following standard procedures (19). EPSP synthase in 50 mM Na-K-phosphate buffer, 1 mM DTT (pH 6.8) was concentrated to 100 mg/ml (about 2 mM) using Centricon 30 concentration devices (Amicon) at 4°C. Crystallization was performed at 20°C using the hanging-drop vapor-diffusion procedure. EPSP synthase was crystallized from 1 M Na-formate/25 mM Na-K-phosphate buffer (pH 7) in the presence of 5 mM S3P or 5 mM S3P and 5 mM glyphosate. Attempts to crystallize EPSP synthase in the absence of S3P under otherwise identical conditions failed.

Diffraction data were collected from flash-frozen crystals by the rotation method and recorded by a RaxisIV detector [x-rays: CuKα, focused by mirror optics (Molecular Structure Corporation, Houston, TX); generator: RU300 (Rigaku)]. Data processing, molecular replacement and refinement, and model building were performed with the program packages XDS (20), CNS

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; PEP, phosphoenol pyruvate; S3P, shikimate-3-phosphate.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID codes 1G6S and 1G6T).

†E.S. and S.E. contributed equally to this work.

‡To whom reprint requests should be addressed. E-mail: eschoenb@eagle.cc.ukans.edu.

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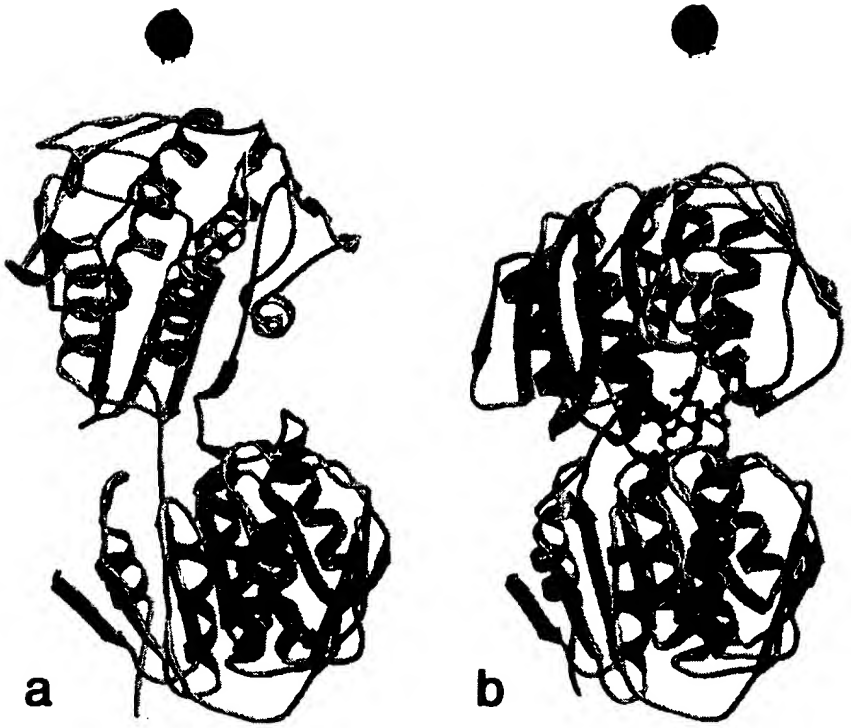

Fig. 1. Cartoon of EPSP synthase in the open and closed conformation. Top domain, residues 20–240; bottom domain, residues 1–19 plus 241–427. (*a*) Unliganded state (open) as reconstructed from the deposited α-carbon atoms (Protein Data Bank entry code 1EPS). (*b*) Liganded state (closed). S3P and glyphosate are shown as ball-and-stick models in green and magenta, respectively. Drawn with BOBSCRIPT (24).

(21), and O (22), respectively. A full-atom starting model of unliganded EPSP synthase was reconstructed with XCHAIN (23) from the deposited Cα atoms (Protein Data Bank entry code 1EPS), stereochemically refined using CNS, and subsequently split into two sets (residues 20–241 and 1–19 plus 242–427). These independent search models for the upper and lower domain of the EPSPS·S3P·glyphosate structure revealed clear signals in the cross-rotational and translational searches with data between 20 and 4.5 Å resolution. After initial rigid-body minimization, all subsequent refinements of the model were performed using data to highest resolution with no σ cut-off applied. Solvent molecules were added to the model at chemically reasonable positions. S3P, glyphosate, and ions were modeled unambiguously into the electron density maps. The EPSPS·S3P structure was solved using the protein part of the final EPSPS·S3P·glyphosate model. Data collection and refinement statistics are summarized in Table 1.

Results and Discussion

Comparing our structures of liganded EPSP synthase with the previously published Cα coordinates of the unliganded enzyme (Protein Data Bank entry code 1EPS; ref. 10), we find that the two domains of EPSP synthase approach each other in a screw-like movement, with the active site emerging in the

Table 1. Summary of data collection and refinement

Crystal	EPSPS·S3P	EPSPS·S3P·glyphosate
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions	a = 58.0, b = 84.9, c = 87.6	a = 57.8, b = 85.2, c = 88.1
Molecules/asym. unit	1	1
Resolution range (Å)	20.0–1.60 (1.65–1.60)	20.0–1.50 (1.55–1.50)
Measured reflections	182207 (12801)	301724 (15899)
Unique reflections	57052 (4837)	67452 (6509)
Completeness (%)	98.7 (95.7)	96.0 (89.2)
R_{sym}* (%)	4.9 (18.1)	3.8 (13.6)
Protein atoms	3232	3232
Alternate atoms positions	58	60
Ligand atoms	16	26
Solvent molecules	555	599
Formate ions	13	10
Phosphate ions	1	0
R_{cryst}† (%)	15.5	15.0
R_{free}‡ (%)	18.6	17.1

Values in parentheses refer to the highest resolution shell.

*$R_{sym} = 100 \times \Sigma_h\Sigma_i|I_{hi} - I_h|/\Sigma_{hi}I_{hi}$ where h are unique reflection indices.

†$R_{cryst} = 100 \times \Sigma|F_{obs} - F_{model}|/\Sigma F_{obs}$ where F_{obs} and F_{model} are observed and calculated structure factor amplitudes, respectively.

‡R factor calculated for 3% randomly chosen reflections, which were excluded from the refinement.

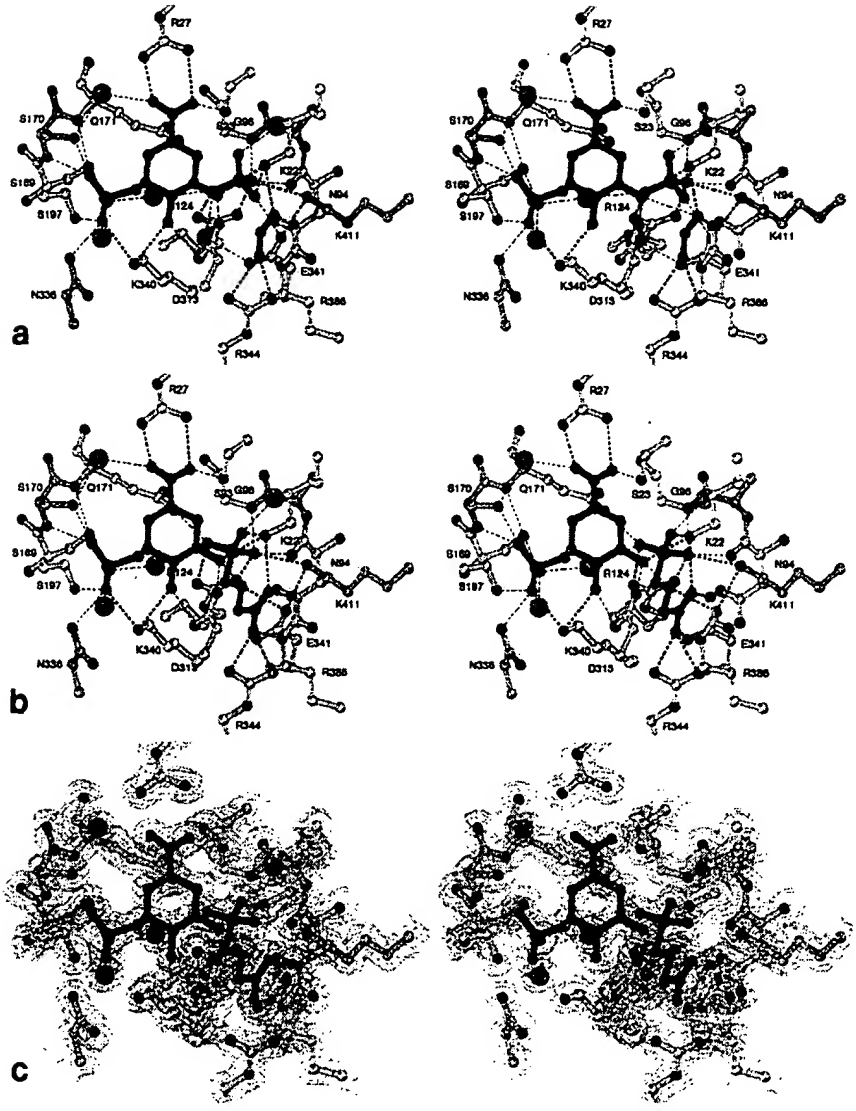

Fig. 2. The active site in S3P-liganded EPSP synthase. Strictly conserved residues are highlighted yellow. Noncarbon atoms are color-coded with blue for nitrogen, red for oxygen, and magenta for phosphorus. Light-blue spheres designate solvent molecules. Hydrogen bonds and ionic interactions are indicated by thin and thick dashed lines, respectively. Tyr-200, which is in hydrophobic contact with the cyclohexenyl moiety of S3P, is not shown. (*a*) In the absence of glyphosate (stereo view). S3P, formate, and phosphate are shown in green. (*b*) In the presence of glyphosate (stereo view). S3P and glyphosate are shown in green. (*c*) Final $2F_o - F_c$ electron density map of the EPSP synthase·S3P·glyphosate complex to 1.5-Å resolution contoured at 1σ (stereo view). Drawn with BOBSCRIPT (24).

interdomain cleft (Fig. 1). Glyphosate binds close to S3P (Fig. 2*b*) without perturbing the structure of the active-site cavity established in our inhibitor-free EPSP synthase·S3P complex (Fig. 2*a*). Distances between glyphosate, S3P, and surrounding protein side chain atoms are in good agreement with those predicted from solid-state NMR data (25, 26). The 5-hydroxyl group of S3P is hydrogen bonded to the nitrogen atom of glyphosate (Figs. 2*b* and 3). Two additional interactions between substrate and inhibitor are mediated through the side chain of Lys-22 and water molecule W2. The glyphosate binding site is dominated by charged residues from both domains of the enzyme, of which Lys-22, Arg-124, and Lys-411 have previously been implicated in PEP binding (27). In the absence of glyphosate from otherwise identical crystallization conditions (see *Methods*), a phosphate and a formate ion (Fig. 2*a*) occupy the positions of the phosphonate and carboxyl groups of glyphosate. However, the overall structure of this EPSP synthase·S3P complex is virtually identical with that of the EPSP synthase·S3P·glyphosate complex, representing the closed state of the enzyme. Because S3P appears to be essential for crystal formation in the used 1 M Na-formate/25 mM Na-K-phosphate (pH 7), we propose that it is S3P that triggers the enzyme's transition from the open to the closed state and not the negatively charged ions or glyphosate. Domain closure would lead to an accumulation of positive charges in the cleft attracting negatively charged molecules to the active site cavity. The proposed ordered mechanism, in which S3P binding is a prerequisite for glyphosate binding, is corroborated by the finding that the substitution of alanine for Arg-27, a residue that interacts exclusively with S3P (Figs. 2 and 3), prevents both the binding of S3P and that of glyphosate (27).

The formate and phosphate ions in the glyphosate-free structure (Fig. 2*a*) are coordinated by the same salt bridges

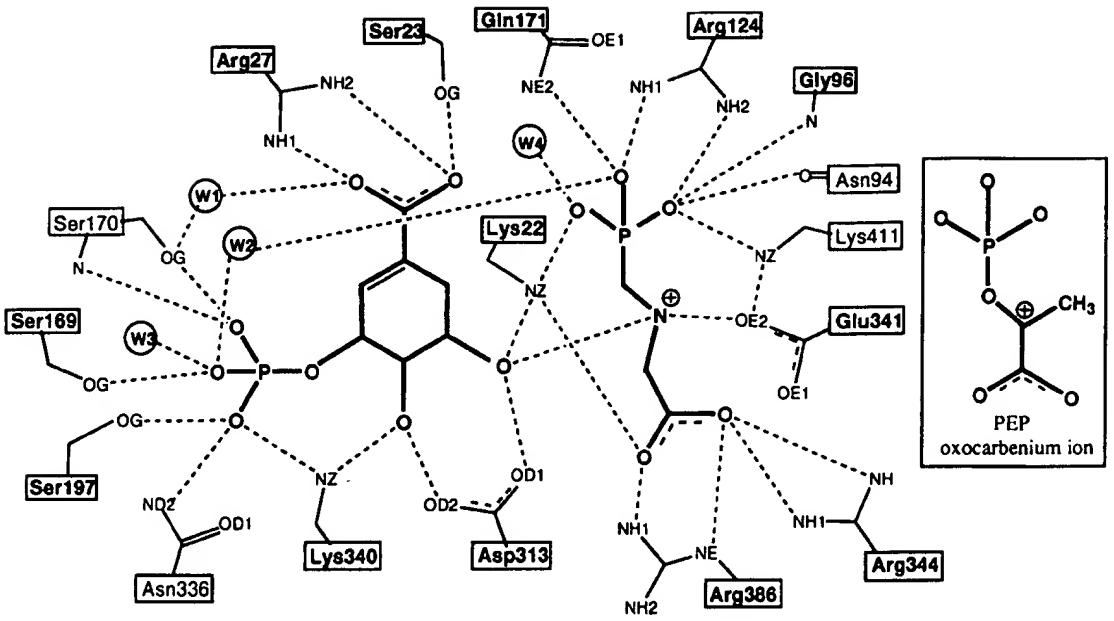

Fig. 3. Schematic representation of ligand binding in the EPSP synthase·S3P·glyphosate complex. Ligands are drawn in bold lines. Dashed lines indicate hydrogen bonds and ionic interactions. Strictly conserved residues are highlighted by bold labels. Protein atoms are labeled according to the Protein Data Bank nomenclature. Circled labels W1 to W4 designate solvent molecules. Hydrophobic interactions between S3P and Tyr-200 are omitted.

that bind the anionic centers of glyphosate (Figs. 2*b* and 3), suggesting that the phosphate and carboxyl moieties of PEP should exhibit the same ionic interactions. Clearly, the anionic centers of PEP are closer to each other than in the extended glyphosate conformation observed in our structure. Assuming that the carboxyl moieties of PEP and glyphosate are in the same position, the side chains of Lys-22, Arg-124, and Lys-411 must follow the phosphate group of the shorter PEP. The crystal structures indicate that the stretching of Arg-124 and slight rearrangements of the two lysine side chains required to maintain the interaction network are easily possible. This proposal is in line with the properties of the glyphosate-tolerant mutant protein Gly96Ala (28). The additional methyl group of the mutant would clash with the phosphonate group of glyphosate but would interfere less with the more remote phosphate group of PEP. Further evidence for a shared binding site between PEP and glyphosate in EPSP synthase comes from structural comparison with the mechanistically related (29) MurA. Comparing the glyphosate binding site of EPSP synthase with that part of the active site of MurA that binds the PEP moiety of the fluorinated analogue of MurA's tetrahedral reaction intermediate (Protein Data Bank entry code 1A2N; ref. 30), we identify the strictly conserved residues Lys-22, Arg-120, Asp-305, Arg-331, Arg-371, and Arg-397, which coordinate the PEP moiety in MurA, as corresponding to Lys-22, Arg-124, Asp-313, Arg-344, Arg-386, and Lys-411, respectively, of EPSP synthase. Thus, our results provide evidence that glyphosate occupies the PEP binding site, and they appear to rule out a recently suggested allosteric action of the herbicide (17, 18).

With the proposed position of PEP, Glu-341 could act as proton donor for the methylene group of PEP; proton addition would then proceed stereospecifically from the 2-*si* face of PEP consistent with previous findings (29). One carboxyl oxygen of Glu-341 could stabilize the incipient PEP oxocarbenium ion, as it would be in sub-van-der-Waals distance to C-2 of the carbocation. The side chain of Glu-341 is held in place through interactions with the backbone nitrogen of Glu-341 and with the side chains of Lys-411 and His-385. His-385 (distance His-385 NE2 – Glu-341 OE2 = 2.85 Å) is likely to function as proton source for Glu-341. Mutations of Lys-411 to Arg (27) and of His-385 to Lys, Ala, Gln, and Asn (31–33) result in a drastic decrease in the catalytic efficiency of the enzyme. The 5-hydroxyl group of S3P has to be deprotonated to attack the C-2 of PEP (34). In our structures, the 5-hydroxyl interacts with Asp-313 and Lys-22. Asp-313 probably acts as proton acceptor, whereas Lys-22 could protonate the oxygen of the scissile bond to facilitate the formation of inorganic phosphate.

The similarity between the two enolpyruvyl transferases EPSP synthase and MurA, pointed out above, appears to extend to details of the induced-fit mechanism. The MurA residues Arg-91, Asp-231, and Asp-369, thought to ease the transition from the open to the closed state of this enzyme (14–16), map to Arg-100, Asp-242, and Asp-384 of EPSP synthase, respectively. As shown for the substitutions R100A, D242A, and D384A (27), mutation of these residues drastically decreases EPSP synthase activity. Because none of these residues is involved in substrate binding, it is possible that the mutations hinder domain closure. By exploiting the analogy between EPSP synthase and MurA, the now available three-dimensional information on these two important drug targets should guide biochemical experiments to further clarify the unusual reaction principles of enolpyruvyl transfer and the associated induced-fit mechanism. In designing novel antimicrobial and herbicidal agents, it could be useful to construct molecules that block domain closure vital for the function of both enzymes.

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Site-directed Mutagenesis of a Conserved Region of the 5-Enolpyruvylshikimate-3-phosphate Synthase Active Site*

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Stephen R. Padgette‡, Diane Biest Re, Charles S. Gasser§¶, David A. Eichholtz, Ronald B. Frazier§, Cathy M. Hironaka, Elaine B. Levine, Dilip M. Shah, Robert T. Fraley, and Ganesh M. Kishore

From the Monsanto Agricultural Company, New Products Division, and §Monsanto Corporate Research, Chesterfield, Missouri 63198

The active site of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) has been probed using site-directed mutagenesis and inhibitor binding techniques. Replacement of a specific glycyl with an alanyl or a prolyl with a seryl residue in a highly conserved region confers glyphosate tolerance to several bacterial and plant EPSPS enzymes, suggesting a high degree of structural conservation between these enzymes. The glycine to alanine substitution corresponding to *Escherichia coli* EPSPS G96A increases the $K_{i(app)}$(glyphosate) of petunia EPSPS 5000-fold while increasing the $K_{m(app)}$(phosphoenolpyruvate) about 40-fold. Substitution of this glycine with serine, however, abolishes EPSPS activity but results in the elicitation of a novel EPSP hydrolase activity whereby EPSP is converted to shikimate 3-phosphate and pyruvate. This highly conserved region is critical for the interaction of the phosphate moiety of phosphoenolpyruvate with EPSPS.

Site-directed mutagenesis of enzymes is a powerful technique for investigating active site structure-function relationships (1). While the technique has been used to verify the roles of active site residues, there are no reports of its use in examining the relatedness of active sites of enzymes from different sources. In this paper, we have used site-directed mutagenesis to establish that the active sites of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)[1] enzymes from bacteria and plants are similar. The x-ray crystal structure of *Escherichia coli* EPSPS has recently been elucidated at 3-Å resolution (2). At the present time, however, the structure of the EPSPS complex with the inhibitor glyphosate is not available. In the absence of detailed structural information on the EPSPS binding site for glyphosate, we have used site-directed mutagenesis to probe the interaction between the enzyme and glyphosate as well as PEP.

The enzyme EPSPS (EC 2.5.1.19) catalyzes the formation of EPSP and phosphate (P_i) from PEP and shikimate 3-phosphate (S3P) (3), in an unusual carboxyvinyl transfer reaction. EPSPS has been extensively studied since it is the target for glyphosate (*N*-phosphonomethyl glycine) (4), the active ingredient of Roundup™ herbicide, widely used for weed and vegetation control (5). Glyphosate is a competitive inhibitor with respect to PEP of EPSPS, and interacts with the $E \cdot S3P$ complex (6). Of the several known PEP-dependent enzymatic reactions, EPSPS is the only enzyme inhibited by glyphosate. The PEP binding region of EPSPS therefore appears to be unique; indeed, EPSPS is the only enzyme which interacts with PEP as an enzyme-substrate complex ($E \cdot S3P$) and not as the free enzyme. In this paper, we have probed the PEP binding region of EPSPS enzymes through site-directed mutagenesis and kinetic analysis.

Identification of the active site of EPSPS has been largely based on chemical modification studies. Lys^{22} and Lys^{340} of *E. coli* EPSPS can be modified by reaction with pyridoxal phosphate (7) and *o*-phthalaldehyde (8), respectively, resulting in inactivation of the enzyme. Arg^{28} and Arg^{131} of petunia EPSPS are modified by phenylglyoxal, the former being essential for enzyme activity (9). Cys^{408} of *E. coli* EPSPS is highly reactive, but not essential for enzymatic activity (10). This residue is proximal to the active site, since its modification with bulky reagents results in inactivation of the enzyme.

Another useful method of active site characterization is identification of substitutions which impact ligand binding and catalysis. Comai and co-workers have described a glyphosate-tolerant *Salmonella typhimurium* strain, wherein the tolerance to glyphosate results from a single amino acid substitution of P101S in the *aroA* gene encoding EPSPS (11, 12). We recently reported the isolation of an *E. coli* B variant, containing a highly glyphosate-tolerant EPSPS (13). Isolation and sequencing of the *aroA* gene encoding this glyphosate-tolerant EPSPS revealed that the altered affinity for glyphosate was the result of a single amino acid substitution of alanine for glycine at residue 96.[2] As shown in Fig. 1, alignment of the amino acid sequences of EPSPS from petunia (14), tomato (14), *Arabidopsis* (15), *Brassica napus*,[3] soybean,[3] maize,[3] *E. coli* (16), *S. typhimurium* (12), *Aspergillus nidulans* (17), *Saccharomyces cerevisiae* (18), and *Bordetella pertussis* (19) revealed that the *E. coli* Gly^{96} residue is located in a highly conserved region of EPSPS, with a consensus sequence $L^{90}XLGNAG^{96}TAXRXL^{102}$ (*X* represents nonconserved

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‡ To whom correspondence should be addressed: Monsanto Agricultural Co., 700 Chesterfield Village Parkway, St. Louis, MO 63198. Tel.: 314-537-6386; Fax: 314-537-6759.

¶ Present address: Dept. of Biochemistry and Biophysics, University of California, Davis, CA 95617.

[1] The abbreviations used are: EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography.

[2] G. M. Kishore, L. Brundage, D. Rochester, and D. Shah, unpublished data.

[3] C. S. Gasser, E. B. Levine, D. M. Shah, C. M. Hironaka, S. Elmer, and H. J. Klee, unpublished data.

TABLE I

Sequence comparison of the conserved region of EPSPS around Gly[96] of E. coli EPSPS

The *underlined* glycine residues correspond to Gly[96] of *E. coli* EPSPS.

EPSPS source	Sequence	Reference
E. coli	L^{90} F LGNA$\underline{G}^{96}$ TAMRP^{101} L^{102}	16
S. typhimurium	L^{90} F LGNA$\underline{G}^{96}$ TAMRP^{101} L^{102}	12
Petunia	L^{95} F LGNA$\underline{G}^{101}$ TAMRP^{106} L^{107}	14
Tomato	L^{95} F LGNA$\underline{G}^{101}$ TAMRP^{106} L^{107}	14
Arabidopsis	L^{95} Y LGNA$\underline{G}^{101}$ TAMRP^{106} L^{107}	15
B. napus	L^{95} Y LGNA$\underline{G}^{101}$ TAMRP^{106} L^{107}	Footnote 3
Soybean	L^{98} F LGNA$\underline{G}^{104}$ TAMRP^{109} L^{110}	Footnote 3
Maize[a]	L^{95} F LGNA$\underline{G}^{101}$ TAMRP^{106} L^{107}	Footnote 3
A. nidulans[b]	L Y LGNA$\underline{G}$ TASRF L	17
S. cerevisiae[b]	L Y LGNA$\underline{G}$ TASRF L	18
B. pertussis	L^{90} F LGNA$\underline{G}^{96}$ TAFRP^{101} L^{102}	19
Consensus[c]	L *X* LGNA$\underline{G}$ TAXRX L	

[a] Based on prediction on start of mature protein (see "Results").
[b] Part of the arom multienzyme complex.
[c] For the consensus sequence, *X* represents nonconserved amino acids.

amino acids). We now show that the glycine to alanine substitution corresponding to *E. coli* EPSPS G96A also imparts glyphosate tolerance to five additional EPSPS enzymes. The substitution corresponding to *S. typhimurium* EPSPS P101S (see Table I) (11, 12), discussed above, also confers glyphosate tolerance to petunia EPSPS. This region is therefore a critical part of an EPSPS active site highly conserved between plant and bacterial enzymes.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All plasmid constructions were carried out by standard methods (20, 21). Oligonucleotide-directed mutagenesis was carried out according to standard methods (22, 23) with minor modifications. Vectors for expression of the petunia, *E. coli*, soybean, and maize enzymes in bacteria utilized the P_L promoter of phage λ (24). The tomato EPSPS bacterial expression vector utilized the *E. coli* RecA promoter (25, 26). In each case the success and fidelity of the mutagenesis was confirmed by sequencing the mutated region (27), using chemically synthesized sequencing primers for an adjacent region.

Enzyme Extraction and Assay—*E. coli* SR481 cultures (aroA deficient) (28) harboring plasmids containing EPSPS genes were grown in Luria-Bertani broth to saturation at 37 °C, centrifuged, washed with 0.9% saline, and resuspended in extraction buffer consisting of 100 mM Tris-Cl, 1 mM EDTA, 10% glycerol, 5 mM dithiothreitol, 1 mM benzamidine HCl, pH 7.5, at 4 °C. The cell suspensions were passed through a French pressure cell (Aminco) twice (1000 p.s.i.), and the resulting lysate was centrifuged (10 min, 10,000 rpm in a Sorvall SS-20 rotor). The supernatant was then passed over a G-25 gel filtration column (Pharmacia LKB Biotechnology Inc., P-10) equilibrated with extraction buffer, and protein concentrations were determined by the method of Bradford (29) using the Bio-Rad microprotein assay. EPSPS was assayed by HPLC radioassay or phosphate release assay as previously described (10) at 25 °C. EPSPS specific activities are reported as μmol of EPSP/min/mg of protein (units/mg). The S3P, [^{14}C]S3P, EPSP, and [1-*enol*-^{14}C]EPSP (used for both EPSP and P_i $K_{m(app)}$ determinations) were synthesized and purified as previously described (28, 30).

Enzyme Purification—EPSPSs were purified as previously described (28), with minor modifications. The pG96S EPSPS[4] was followed during purification by HPLC with a Vydac C18 column, using 45–85% RP-B in 20 min (RP-A, 0.1% trifuoroacetic acid; RP-B, 0.1% trifluoroacetic acid in acetonitrile) at 1 ml/min flow rate; the EPSPS retention time was 14 min.

Equilibrium Dialysis—For binding studies, enzymes were dialyzed at 5 °C into dialysis buffer (50 mM HEPES, 50 mM KCl, 10% glycerol, 5 mM β-mercaptoethanol, pH 7.0) (2 × 2 liters, 4 h). After dialysis, the concentration was adjusted to 1.0–1.8 mg/ml with dialysis buffer (using $A_{280}^{1\%} = 8.2$). The experiments (duplicate) were performed by dialyzing a fixed amount of enzyme against increasing concentrations of [^{14}C]S3P in a microdialyzer apparatus (Hoefer, 250-μl chambers). S3P solutions (15–260 μM) were prepared by mixing [^{14}C]S3P (19.7 mCi/mmol) with increasing amounts of unlabeled S3P. The enzyme (200 μl) was placed into one half-cell and an equal volume of S3P was placed into the other half-cell; dialysis cells were rotated at 20 rpm for approximately 16 h at 5 °C. Aliquots (100 μl) were removed from each half-cell, and the radioactivity present at equilibrium was determined by liquid scintillation counting (Beckman LS). The radioactivity present in the half-cell containing protein was used to calculate the concentration of bound + free S3P. The radioactivity present in the half-cell without protein was used to calculate the concentration of free S3P. The data was then plotted as $1/[S3P]_{free}$ *vs.* $[EPSPS]/[S3P]_{bound}$ to determine K_d, the equilibrium dissociation constant (31).

RESULTS AND DISCUSSION

Construction of EPSPS Expression Vectors—We have previously described a system for the production of a mature (minus chloroplast transit peptide) form of petunia EPSPS in *E. coli* (28). The codon for Lys[73] of petunia preEPSPS (14) that represents the first amino acid of the mature protein was replaced by oligonucleotide-mediated mutagenesis with two codons, Met and Glu, producing a convenient restriction enzyme recognition site that allowed for correct attachment of the coding sequence to a strong *E. coli* promoter and ribosome binding site (24). For the current study, we have constructed similar vectors for bacterial expression of mature forms of EPSPS from tomato, soybean, and maize. In tomato and soybean the high degree of identity of the EPSPSs to petunia EPSPS allowed for identification of the homologous Lys residue as the putative N terminus of the mature EPSPS, which was altered as described above and inserted into *E. coli* expression vectors. Our best prediction of the maize CTP cleavage site was between Glu[62] and Ala[63] of the maize preEPSPS.[5] In this case mutagenesis was performed to add a codon for a Met residue just upstream of the Ala[63] codon. The altered maize sequence was then used to construct a vector for expression in *E. coli*. The bacterial expression plasmids were introduced into an aroA-deficient *E. coli* strain, SR481, which does not produce an active EPSPS, as previously described (28). A genomic clone for *Arabidopsis* EPSPS has previously been used to engineer high level expression of EPSPS in transgenic *Arabidopsis* plants (15). Plants produced using this system provided a source for the *Arabidopsis* EPSPSs.

To determine the effect on the plant EPSPSs of the glycine to alanine substitution corresponding to *E. coli* EPSPS G96A, we introduced the corresponding substitution by site-directed mutagenesis into the EPSPS DNA clones for bacterial expression of the petunia, tomato, soybean, and maize variants, and for plant expression of the *Arabidopsis* variant (Table I). Because of differences in amino acid sequence numbering, the *E. coli* EPSPS G96A substitution corresponds to different Gly to Ala substitutions in plant EPSPSs (for instance, G101A in

[4] To avoid confusion, the numbering of all EPSPS amino acid substitutions in this paper will be based on the *E. coli* EPSPS sequence. To denote different gene sources, a code letter will precede the substitution designation. The following gene source designation codes are used: petunia, "p"; tomato, "t"; maize, "m"; *Arabidopsis*, "a." Therefore, the Gly to Ala substitution corresponding to G96A in *E. coli* EPSPS will be denoted pG96A in petunia EPSPS. Similarly, specific residues are preceded by their origin code, for instance $pGly^{96}$ of petunia EPSPS. Note that the conserved region containing this residue has the same numbering in both *E. coli* and *S. typhimurium* EPSPS.

[5] C. S. Gasser, unpublished data.

petunia EPSPS), although as shown in Table I, the residues are homologous. The amino acid numbering system used herein is based on the *E. coli* EPSPS numbering scheme.[4]

Analysis of Wild-type and Ala Variant EPSPSs—The wild-type and Ala variant EPSPSs were extracted from *E. coli* cells or *Arabidopsis* leaf tissue and assayed for EPSPS activity. The wild-type plant EPSPSs had specific activities ranging from 0.18 to 1.6 units/mg, compared to 0.11–0.69 unit/mg for the corresponding Ala variants (Table II). There does not appear to be any significant alteration in EPSPS specific activity resulting from the engineering of the Ala substitution corresponding to *E. coli* EPSPS G96A, except perhaps in the tG96A variant, which only exhibits about 25% of the specific activity of the tomato wild-type enzyme. Manipulation of residues immediately around the chloroplast transit peptide cleavage site does not appear to significantly impact the enzyme activity of the mature EPSPSs, probably because this region folds into a domain separate from the mature region of preEPSPS (32). All of the plant EPSPS cDNAs complemented the *aroA* mutation in *E. coli* SR481.

Crude extracts containing either the wild-type or Ala-substituted variant EPSPSs corresponding to G96A were also assayed for their glyphosate sensitivity at saturating substrate concentrations. All the wild-type EPSPSs tested were sensitive to inhibition by glyphosate, while the Ala-substituted variants were extremely resistant to inhibition by glyphosate (Table II). Thus, the substitution corresponding to G96A affects the glyphosate sensitivities of both *E. coli* and plant EPSPSs to similar degrees, with more than a 500-fold increase in IC_{50} being obtained in all cases. This newly introduced Ala residue therefore interferes with glyphosate binding to EPSPS by a mechanism common to all EPSPSs tested, and furthermore, these results indicate that the active site of EPSPS is highly conserved among the plant and bacterial enzymes.

Steady-state Kinetic Analysis of pG96A Petunia EPSPS—In order to better quantitate the effect of the pG96A substitution on petunia EPSPS, steady-state kinetic constants were determined for purified wild-type and pG96A petunia EPSPSs (Table III). For the pG96A variant, the $K_{i(app)}$(glyphosate) *vs.* PEP was found to be 2.0 ± 0.2 mM, which is 5000 times greater than that of the wild-type enzyme; the inhibition remained competitive *vs.* PEP. The $K_{m(app)}$(PEP) is elevated from 5.0 ± 1.3 μM in the wild-type enzyme to 210 ± 20 μM in the pG96A variant, and the $K_{m(app)}$(phosphate) is elevated from 0.59 ± 0.09 mM for the wild-type enzyme to 6.7 ± 1.2 mM for the pG96A variant. These kinetic constants for the pG96A EPSPS are similar to those reported for the G96A *E. coli* B EPSPS ($K_{i(app)}$(glyphosate) = 4.1 mM, $K_{m(app)}$(PEP) = 220 μM) (13). In contrast to the decreases in apparent binding seen for glyphosate, PEP, and P_i in the pG96A variant, the $K_{m(app)}$ values for both S3P and EPSP are essentially unchanged between the wild-type and pG96A EPSPSs (Table III). The $K_{m(app)}$ values determined for the wild-type petunia enzyme are similar to the experimentally determined ligand dissociation constants for *E. coli* EPSPS (K_d(S3P) = 7 μM, K_d(PEP) = 18 μM, K_d(glyphosate) = 0.16 μM, K_d(EPSP) = 1 μM, and $K_d(P_i)$ = 1.4 mM for *E. coli* EPSPS) (33), indicating that, for EPSPS, measurement of $K_{m(app)}$ values is a valid approximation to the ligand dissociation constants. Taken together, these kinetic data are consistent with a perturbation of a binding interaction common for glyphosate, PEP, and P_i, but not S3P or EPSP. Since glyphosate is a competitive inhibitor of EPSPS with respect to PEP, it is not surprising that the pG96A variant (and the *E. coli* EPSPS G96A variant) also displays a loss of PEP binding. If one views the $K_{i(app)}$(glyphosate)/$K_{m(app)}$(PEP) as a selectivity factor for PEP over glyphosate binding, the large increase in its value from the petunia wild type EPSPS (K_i/K_m = 0.08) to the pG96A variant (K_i/K_m = 9.5) indicates that the introduction of the alanine residue selectively destabilizes the interaction of the enzyme with glyphosate compared to PEP.

In addition to its glyphosate insensitivity, the catalytic efficiency for pG96A EPSPS is also altered; the k_{cat} in the forward reaction direction is 58% of that of the wild-type enzyme. The $k_{cat}/K_{m(app)}$(PEP) values for the wild-type and pG96A EPSPSs were 7.2 $s^{-1}\mu M^{-1}$ and 0.1 $s^{-1}\mu M^{-1}$, respectively, indicating that the pG96A variant is about 72 times less efficient than the wild-type enzyme. The relative catalytic efficiency in the presence of glyphosate, $k_{cat} \cdot K_{i(app)}$-(glyphosate)/$K_{m(app)}$(PEP), indicates that the pG96A variant is about 69 times more efficient than the wild-type enzyme in the presence of glyphosate.

Interaction of pG96A Petunia EPSPS with a Reaction Intermediate Analog—Alberg and Bartlett (34) have synthesized a potent EPSPS inhibitor, 5-*O*-[(*R*)-1-carboxy-1-phosphonoethyl]shikimate 3-phosphate, which mimics the tetrahedral reaction intermediate (35) and inhibits petunia EPSPS with a K_i of 15 nM (34). In order to determine if pG96A EPSPS efficiently interacts with the tetrahedral intermediate analog, we determined the $K_{i(app)}$ for the *R*-inhibitor for pG96A and wild-type petunia EPSPS in the forward reaction direction. Interestingly, the analog inhibited pG96A EPSPS with an $K_{i(app)}$ of 35 ± 2 nM, compared to 38 ± 5 nM for the wild-type enzyme (S3P = 2 mM; PEP = 5 μM for wild-type and 40 μM for pG96A EPSPS). The petunia EPSPS pG96A substitution therefore does not adversely affect the interaction of the phosphonate moiety of the analog with EPSPS and is in agreement with our observation that the pG96A variant has a V_{max} close to that of the wild-type enzyme, presumably due to its high affinity for the reaction intermediate.

pG96S Petunia EPSPS—The $pAla^{96}$ substitution in the pG96A petunia EPSPS variant clearly diminishes both glyphosate and PEP binding to the enzyme, albeit to different degrees. If this perturbation was a direct result of the increased steric bulk of the $pAla^{96}$ methyl group compared to the $pGly^{96}$ hydrogen of the wild-type enzyme, one would

TABLE II

Expression of wild-type and Ala variant EPSPSs

The substitutions shown are at the Gly residues underlined in Fig. 1 and correspond to G96A of *E. coli* EPSPS.

EPSPS[a]	Specific activity		IC_{50} (glyphosate)[b]	
	Wild-type	G96A variant	Wild-type	G96A variant
	units/mg		*mM*	
Petunia	0.79	0.69	0.030	17
E. coli	8	6	0.032	76
Tomato	1.6	0.39	0.006	32
Arabidopsis[c]	0.18	0.11	0.037	19
Soybean	0.18	0.22	0.025	18
Maize	0.13	0.16	0.014	9

[a] Enzymes were extracted and assayed as described under "Experimental Procedures."

[b] At least six glyphosate concentrations were used at 1 mM PEP and 2 mM S3P.

[c] Leaf tissue (1 g) from transgenic *Arabidopsis* plants transformed with pMON 599 (overexpressing wild-type *Arabidopsis* EPSPS) (15) or pMON 600 (aG96A variant) was frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle, and 1 ml of extraction buffer (with the addition 5 mM ascorbate and 1 mg/ml bovine serum albumin) was added. After further grinding (45 s), the suspension was centrifuged in a microcentrifuge for 5 min. The resulting supernatants were desalted over spin G-50 columns equilibrated with the same buffer and assayed for EPSPS activity by HPLC radioassay (10).

TABLE III

Steady-state kinetic analysis of pG96A EPSPS

Kinetic constants were determined by HPLC radioassay as previously described (10), using 5–11 duplicated substrate concentrations. Apparent K_m values were determined by computer fit of the data to the hyperbolic form of the Michaelis-Menton equation (nonweighted), ± the standard error of the value derived from the fit (RS/E, BBN, Cambridge, MA); the value in parentheses is the number of independent experiments. Values for $K_{i(app)}$(glyphosate) were determined using an inverse plot at three glyphosate concentrations. Slope replots gave the $K_{i(app)}$(glyphosate) as the x intercept. The $K_{m(app)}$(PEP) was determined at 2 mM S3P, the $K_{m(app)}$(S3P) was determined at 1 mM PEP, the $K_{m(app)}$(EPSP) was determined at 10 mM KP_i, and the $K_{m(app)}(P_i)$ was determined at 100 μM EPSP concentrations. For k_{cat} calculations, the molecular mass of petunia EPSPS used was 47.6 kDa.

EPSPS	$K_{i(app)}$(glyphosate)	$K_{m(app)}$(PEP)	$K_{m(app)}$(S3P)	$K_{m(app)}$(EPSP)	$K_{m(app)}(P_i)$	Specific activity	k_{cat}
	μM	*μM*	*μM*	*μM*	*mM*	*units/mg*	s^{-1}
Wild-type	0.40 ± 0.16	5.0 ± 1.3 (2)	7.8 ± 1.4 (2)	1.9 ± 0.4 (2)	0.59 ± 0.09 (2)	45	36
pG96A	2000 ± 200	210 ± 20 (3)	11 ± 3.3 (2)	2.5 ± 0.3 (3)	6.7 ± 1.2 (2)	27	21

predict that increasing the size of the side chain of the amino acid residue at this position would affect both glyphosate and PEP binding to EPSPS. Therefore, the pG96S EPSPS variant was constructed and assayed in *E. coli* SR481 for its ability to complement the *aroA* deficiency; no complementation was observed. Using *E. coli* extracts containing the pG96S EPSPS (Western blot analysis), we were unable to detect any EPSPS activity. This result suggested that the pG96S substitution may have significantly affected the binding of PEP to the enzyme. If this were the case, S3P binding should remain intact, although the enzyme is catalytically inactive. Indeed, equilibrium dialysis binding experiments using purified pG96S EPSPS established that the K_d(S3P) was 6.0 ± 0.6 μM, compared to 15 ± 3 μM for wild-type petunia EPSPS. Thus, S3P binding remains unaltered in the pG96S variant. However, the pG96S·S3P complex was unable to bind glyphosate, based on fluorescence emission and equilibrium dialysis analyses (data not shown). These features distinguish the pG96S variant from the wild-type enzyme.

The intensity of the fluorescence emission spectrum of pG96S EPSPS did not increase upon EPSP addition, whereas a similar treatment of wild-type enzyme did result in a fluorescence emission spectrum intensity increase (36). HPLC analysis of a pG96S/EPSP mixture, after the addition of 10 mM potassium phosphate, indicated that the added EPSP had been hydrolyzed to S3P and an unknown product, while an identical reaction with wild-type petunia EPSPS gave the expected S3P and PEP product peaks. Additional experiments confirmed these results and showed that phosphate was not required for pG96S-catalyzed EPSP hydrolysis (Fig. 1). The most likely fate of the EPSP carboxyvinyl carbon fragment was postulated to be its conversion to pyruvate; EPSPS has been shown to form pyruvate from S3P and PEP as a by-product at an exceedingly low rate (0.00047 s^{-1} for *E. coli* EPSPS) (33). By using lactate dehydrogenase/NADH, we found that pyruvate is indeed the second product of pG96S-mediated hydrolysis of EPSP. The EPSP hydrolase activity is not sensitive to inhibition by glyphosate, but is highly sensitive to inhibition by S3P (Table IV). In addition, 0.2 mM of the *R*-isomer intermediate analog of the EPSPS reaction described above did not inhibit the pG96S hydrolase reaction (at 57 μM EPSP), while inhibiting 94% of the wild-type petunia EPSPS activity at 57 μM EPSP and 50 mM KP_i. These results confirm our hypothesis that the $pGly^{96}$ region of the petunia EPSPS active site participates in the recognition of the phosphate moiety of PEP. The phosphate recognition site in the pG96S variant is severely disrupted and results in a loss of binding of the intermediate analog, and by inference, the tetrahedral intermediate itself.

There are three possible mechanisms by which EPSP can

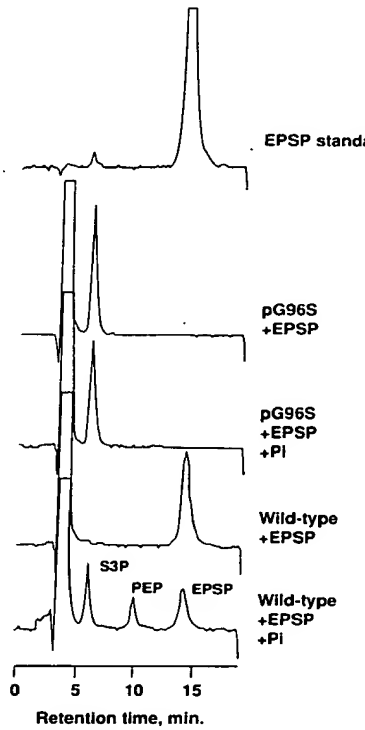


FIG. 1. **pG96S EPSPS reaction with EPSP.** Reactions contained 30 μg of the appropriate EPSPS, 1 mM EPSP, and 50 mM HEPES, pH 7.0, in 100 μl of total reaction volume. Phosphate (P_i) was used at a concentration of 50 mM. Reactions were run for 25 min, then quenched with 100 μl of 90% ethanol, 0.1 M acetic acid, pH 4.5. Aliquots of 50 μl were analyzed by HPLC as previously described (10), using UV detection at 210 nm (0.08 absorbance units at full scale). The EPSPS standard was prepared in water.

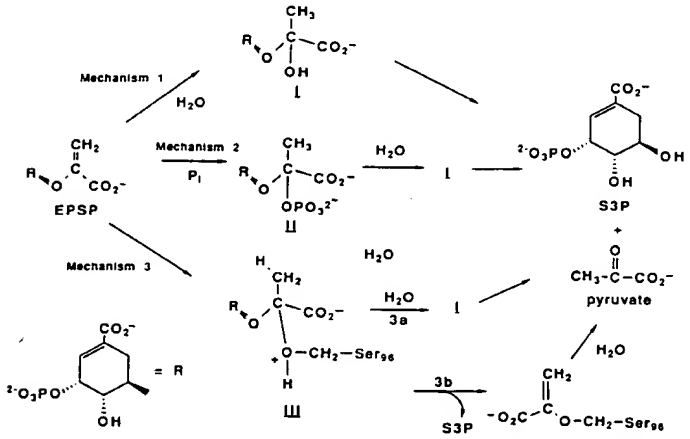
be hydrolyzed by pG96S EPSPS. The first possibility (Scheme 1, mechanism 1) is that nucleophilic addition of water to the C-2 carbon of the carboxyvinyl moiety of EPSP generates a tetrahedral intermediate *I* ,which by ketonization of the C2-OH bond yields pyruvate and S3P. The tetrahedral intermediate *I* is the dephosphorylated form of tetrahedral intermediate *II*, an intermediate of the EPSPS reaction (35). A second possibility is that the phosphorylated tetrahedral intermediate *II* is hydrolyzed by the pG96S enzyme to the intermediate *I* which then follows the same route suggested for mechanism 1. A third possibility is the direct nucleophilic attack of the pG96S $pSer^{96}$ hydroxyl group on the C-2 carbon of the carboxyvinyl moiety of EPSP (mechanism 3), generating an enzyme-bound intermediate which is hydrolyzed to S3P and pyruvate. It is known that the enzyme UDP-*N*-acetyl mur-

TABLE IV

Pyruvate formation by purified pG96S

Reaction mixtures (1.0 ml) contained 0.105 mg of pG96S, 100 μM NADH (Sigma), 80 units of lactate dehydrogenase (Sigma type V-S), and 50 mM HEPES, pH 7.0, with the additional components indicated. After 5-min preincubation at 30 °C, reactions were initiated with 52.5 μM EPSP. Initial rate absorbance measurements at 340 nm were determined at 30 °C on a Hewlett Packard 8452 spectrophotometer, coupling NADH oxidation to pyruvate reduction by LDH. The NADH extinction coefficient used was 6300 M^{-1} cm^{-1} (39).

Number	Additions	Rate	Uninhibited rate
		units/mg	%
1	No additions	0.52	100
2	2.0 mM S3P	0.009	1.7
3	25 mM glyphosate	0.44	85



SCHEME 1. **Mechanisms for pG96S EPSP hydrolase activity.**

amic acid synthase reacts with PEP generating an intermediate with covalently bound *enol*-pyruvate (37). Reaction mechanism 3 is similar except that EPSP replaces PEP as a substrate and water can release the enzyme-bound *enol*-pyruvate. Mechanisms 1 and 3 can be distinguished from mechanism 2 by the P_i dependence of the EPSP hydrolysis reaction of pG96S EPSPS. Trace amounts of phosphate present in the reaction mix were eliminated by the use of the sucrose phosphorylase/sucrose trap system (30). No differences in reaction rates were seen in the presence or absence of the trap system. There was no change in the rate of EPSP hydrolysis when the P_i concentration was increased from 4 μM (background concentration) to 4 mM. These features, plus the lack of inhibition by 5-*O*-[(*R*)-1-carboxy-1-phosphonoethyl]shikimate 3-phosphate, suggest that the EPSP hydrolysis by pG96S operates by the sequence shown in either mechanism 1 or 3. The conversion of intermediate *I* to S3P and pyruvate may also occur by two different mechanisms, either direct dissociation into S3P and pyruvate *or* formation of S3P and *enol*-pyruvate which tautomerizes to pyruvate. Further distinctions between reaction mechanisms can be made by establishing if *enol*-pyruvate is the product of the reaction and if the enzyme catalyzes EPSP-S3P exchange of the enolpyruvyl moiety.

In order to further characterize the pG96S variant, the $K_{m(app)}$(EPSP) relative to that of the wild-type enzyme was determined using the pyruvate kinase/ADP coupled assay. The $K_{m(app)}$(EPSP) of pG96S was 23 ± 6 μM, compared to 20 ± 5 μM for the wild-type EPSPS (10 mM phosphate, spectrophotometric assay at 30 °C (34)). The V_{max} for the EPSP hydrolase activity of pG96S was 1.24 unit/mg, yielding a k_{cat} of 0.98 s^{-1}, which is > 2000 times faster than the rate of EPSP hydrolysis catalyzed by wild-type *E. coli* EPSPS (33). Taken together, these results clearly demonstrate that $pSer^{96}$ of pG96S does not interfere with the S3P/EPSP binding site, but perturbs the PEP binding site. Since the carboxyvinyl moiety of EPSP interacts with the enzyme, the inability of pG96S EPSPS to bind PEP may be most likely due to the steric hindrance for interaction of phosphate moiety of PEP at the active site. It is tempting to speculate that the $pSer^{96}$ hydroxymethyl group of pG96S petunia EPSPS displaces the phosphate of PEP and functions as a nucleophile, attacking the C-2 carbon of the carboxyvinyl moiety of EPSP as suggested in mechanism 3. If this were to be the case, we would predict that variants of EPSPS containing alanine or valine at Gly^{96} should be ineffective in catalyzing this reaction. In accord with this, purified G96A *E. coli* EPSPS did not have detectable EPSP hydrolase activity. Additional experiments are in progress to determine the hydrolase reaction mechanism of pG96S EPSPS.

pP101S EPSPSs—Yet another substitution that has been described in the conserved region of EPSPS is that of P101S in *S. typhimurium* EPSPS (11, 12). Since this proline is not conserved in all EPSPSs (Table I), it was of interest to determine if the corresponding proline to serine substitution would result in a glyphosate-tolerant petunia EPSPS. The pP101S EPSPS cDNA was therefore constructed by site-directed mutagenesis and expressed in *E. coli* SR481. Kinetic analysis of the purified enzyme showed that the $K_{m(app)}$(PEP) was 44 ± 3 μM, the $K_{m(app)}$(S3P) was 12 ± 2 μM, the $K_{i(app)}$(glyphosate) was 3.0 ± 0.8 μM, and the specific activity was 40 units/mg. These results show that for petunia EPSPS, the pP101S substitution results in a decrease in glyphosate binding, the magnitude of which is, however, significantly lower than for the pG96A substitution. The results of these mutagenesis experiments further support the view that the active sites of bacterial and plant EPSPSs are highly conserved.

A doubly substituted petunia EPSPS variant of pG96A pP101S was constructed in order to determine the effect of combining the two single substitutions which independently confer glyphosate tolerance to EPSPS. The pG96A pP101S variant had an $K_{m(app)}$(PEP) of 390 ± 40 μM and an $K_{i(app)}$(glyphosate) of 8.2 ± 0.4 mM. Thus, the addition of the pP101S substitution to the pG96A variant increases the $K_{i(app)}$(glyphosate) by a factor of 4.1 and the $K_{m(app)}$(PEP) by a factor of 1.8. This increase in $K_{i(app)}$(glyphosate) is similar to the increase observed for the pP101S single variant relative to wild-type EPSPS (6-fold) and suggests that the pG96A and pP101S substitutions impart glyphosate tolerance by affecting distinct interactions between glyphosate and the conserved region. The $K_{m(app)}$(PEP) may be somewhat obscure since the absolute K_m(PEP) has not been obtained. Nevertheless, the results of these studies suggest multiple interactions between PEP/glyphosate and the conserved region of EPSPS. One of these interactions may involve the guanidinium side-chain of Arg^{100}, a conserved residue in all EPSPS enzymes (Table I). Unpublished experiments from our laboratory based on mutagenesis of $pArg^{100}$ support this hypothesis.[6] A complete understanding of the nature of these interactions will have to await the x-ray structure elucidation of the enzyme·substrate/inhibitor complexes.

It is clear that considerable information about the active site of EPSPS has been obtained by site-directed mutagenesis and kinetic characterization of the variant enzymes. Recently, the x-ray structure of wild-type *E. coli* EPSPS has been elucidated at 3-Å resolution (2). The EPSPS polypeptide has

[6] S. R. Padgette, D. B. Re, C. M. Hironaka, and G. M. Kishore, unpublished data.

a two-domain structure with a novel fold that appears to be formed by a 6-fold replication of a protein folding unit comprised of two parallel helices and four stranded sheets. In this structure, the active site appears to be in the interdomain region, held apart by the interdipole repulsions in the absence of the anionic ligands. Since significant conformational changes can be demonstrated upon interaction of EPSPS with either S3P and glyphosate or EPSP and glyphosate, the structure of the native enzyme is insufficient to explain the results obtained with the variant enzymes. However, our knowledge of the variant enzymes can be of great value toward building models for the structure of the enzyme complexes. For a model to be valid, it must account for the perturbations in the kinetic constants of the variants described herein as well as the EPSP hydrolase activity of the pG96S variant. It is interesting to note that the two primary substitutions that confer glyphosate tolerance to EPSPS are localized within the conserved region shown in Table I. Whether this is the only region of EPSPS that can provide differentiation between PEP and glyphosate binding needs to be determined either by elucidation of the x-ray structure of an EPSPS·glyphosate (or PEP) complex or isolating additional glyphosate-tolerant EPSPS variants.

In summary, we have utilized site-directed mutagenesis to identify the active site of EPSPS. These studies have established that the conserved region of EPSPS containing Gly^{96} is critical for interaction with the phosphate moiety of PEP, inorganic phosphate, and the phosphonate of glyphosate. The glyphosate-tolerant EPSPS cDNA clones have been used for conferring *in planta* Roundup™ tolerance to crop plants (38). The levels of tolerance are significantly higher than that achieved using the wild-type, glyphosate-sensitive EPSPS enzymes. Understanding the molecular basis of tolerance will further facilitate new inhibitor design and the understanding of the catalytic mechanism of the enzyme.

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